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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The main purpose of our work is to determine the expression pattern of TLKs and Rad9 in prostate cancer cell lines and in patients' biopsies, and their relation to radiation resistance and (consequently) XRT failure. A second project was to identify compounds that inhibit the activity of TLKs and its phosphorylation of Rad9, and then determine the in vitro consequences of applying these compounds to the pattern of radiation sensitivity. A final goal, which is not really part of the listed tasks, is to understand better the mechanism of action of TLKs in DNA DSB repair. This is essential for the application of the use of the radiosensitizers one day to a human clinical trial, as basic information on their mechanism of action is critical. We have published the expression TLKs and Rad9 in prostate cancer cell lines (see attached PDF). We have also published two papers on the mechanism of action of TLKs and DSB repair (see attached). We have begun analyzing TMAs for the pattern of expression of TLKs and Rad9. This is in collaboration with MSKCC. We have probed the first TMA, but are waiting for quantitative analysis with the ARIOL system. We do have (indirectly) information on the patients, as per our approved IRB. We plan to analyze one or two more TMAs in the next fiscal year before we can make solid conclusions on patients' failure to XRT. We have identified 4 structurally-related compounds from our screen (~5000 clinically-relevant compounds) that are strong inhibitors of TLKs. These are certain interesting phenothiazines, and we are now studying their activity in a panel of CaP cell lines. They do synergize in killing with IR or doxorubicin, and they inhibit Rad9 (S328) phosphorylation – in a direct in vitro reaction with recombinant TLK and Rad9 peptide, or in cell lines treated with doxorubicin.

15. SUBJECT TERMS
Tousled like kinases, Rad9, radiation-resistant prostate cancer, radiosensitizers

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Year 1:

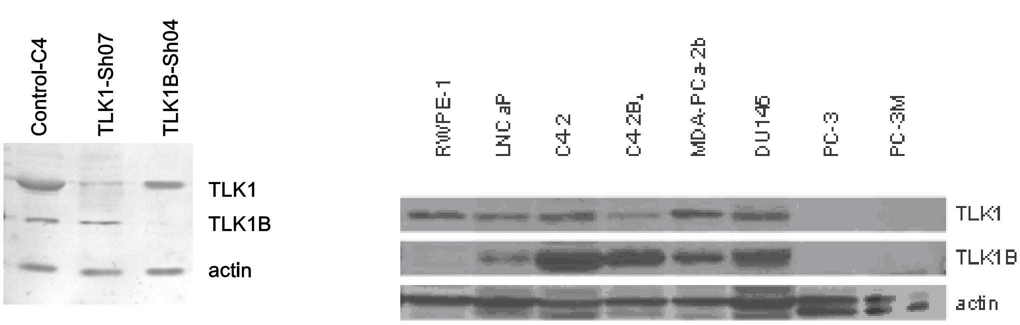
- Immunostaining of previously collected tissue samples for eIF4E, TLK1, TLK1B, and Rad9 expression and correlation with clinical parameters.
- • Examine by western blot total TLK1B and Rad9 in CaP cell lines
- o Examine by western blot nuclear and cytoplasmic partitioning of TLK1B and Rad9 in CaP cell lines
- • Determine correlation between TLK1B and Rad9 expression and radioresistance
  - o Extract protein from nucleus and cytoplasm ± IR
  - o Perform WB

Years 1-2: Determination of the role of TLK1B in localization of Rad9 at DSBs

We have probed the first TMA of patients who have failed XRT and have gone for salvage prostatectomy (From MSKCC patients), and have probed two in-house TMAs. We have to analyze the same TMAs now for TLK2 9the second Tousled gene in humans0, as this turned out to be equally important (see the Prostate 2011 paper), and the Rad9 phosphorylation picture is incomplete w/o this information. All of this massive amount of microscopic data need then to be quantified with the ARIOL system. It will probably take the next fiscal year and the probing of one or two more TMAs to be able to reach final determinations.
We have completed the analyses of TLKs expression in the most common human CaP cell lines (see the Prostate 2011 paper). Rad9 expression and co-localization studies with TLKs are in progress, along with actual tissue sample, as indicated above.

Months 1-6:

• make and test the shRNA retroviruses to knock down TLK1B.
We have made the shRNA retroviruses and they work (see WB below)



Effect of shRNA on TLK1 and TLK1B expression. C4 prostate cancer cells that express TLK1 and TLK1B were infected at an MOI of 50, and after 12h, extracts were analyzed by western blot for TLK1/1B. **B. Western blots of TLK1, TLK1B** in prostate cell lines.

Months 7-18:

- determine the role of TLK1B in assembly of Rad9 both on IR-induced DSBs by immunofluorescense
- determine the role of TLK1B in assembly of Rad9 both on a single HO-induced DSB by ChIP

We are currently conducting this work. An initial report was published in Sunavala-Dossabhoy G, and De Benedetti A. (2009) Tousled homolog, TLK1, binds and phosphorylates RAD9 – TLK1 acts as a molecular chaperone in DNA repair. DNA Repair 8(1):87-102.

Years 1-3:

• Screening of compound libraries and analysis of cell lines for the discovery of radiosensitizers

Year 1:

• prepare and validate primary assay

- o screen all 11,000 compounds
- o identify hits

Year 2: Conduct counterscreens

- 6 point dose-response
- IR-mediated cell survival assays
- identify leads

Year 3: Test leads
- Genotoxicity assays; cancer cells, normal epithelium, liver, and kidney cell lines
- Cell surivival
- Soft agar colony formation

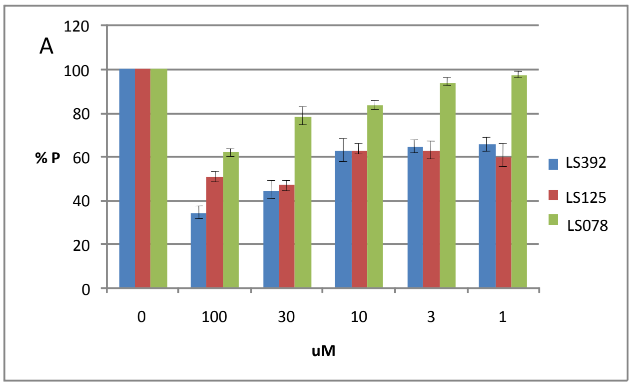
We have made much progress on this as detailed below. This is proprietary information and the compounds are not directly identified here, but some are common antipsychotic drugs, hence immediately usable as they are FDA-approved, such as Triflorperazine. This compound was found previously (accidentally) to inhibit DSB repair and radiosensitize cells (see ref. in the list below).

Preliminary Results. Identification of TLK inhibitors. We have screened the Prestwick library and two other proprietary libraries (~5000 compounds) for inhibitors of TLK with a high throughput phosphorylation screen using recombinant TLK1B and a Rad9 peptide as the substrate - details of the screen are proprietary at this stage. We have identified 4 strong inhibitors that are structurally and chemically very similar: they will be known as LS-266 ("class lead"), LS-125, LS-078 and LS-392. The inhibition of phosphorylation was confirmed by TCA-precipitable counts with γ^{32}P-ATP (Fig. 1A). The inhibitors caused markedly increased sensitivity to IR and doxorubicin (Fig. 3) that could be explained by inhibition of NHEJ. This was shown as slower regression of DSB repair foci (γH2AX - not shown), and delayed repair kinetics of the single DSB generated with HO endonuclease (Fig. 2). The inhibitors are highly specific. A commissioned KinomeScan (DiscoverX) with LS078 revealed that no other kinase in the panel was inhibited, and the compounds don't resemble ATP. Fig. 1B shows that the drugs worked specifically at low μM concentration after immunoprecipitation (IP) of TLK1 from 293T cells, and interestingly, they remained associated with the protein, retaining their inhibition even after removal from the medium - After IP we did not add the drugs back in the autophosphorylation reaction. As shown in Fig. 2, repair the HO- DSB, determined as loss and then reappearance of the amplicon was much slower after adding LS-125.

Work in Year 3 on the TLK inhibitors on tissue culture cells will be moved to year 2 since we are ahead of where we thought we would be. We then foresee to conduct some animal work in year 2-3, but we don't need to transfer funds or file a rebudget, since we have a "free" SCID mice colony that is maintained by our Cancer Center (FWCC), and there are also some funds available to us members of the FWCC. Institutional Animal approval protocols are not directly tied to grants or sponsored projects.

"so what section": The identification of TLK inhibitors offers the promise to approach XRT as the treatment of choice for first-line CaP "cure", as they will be extremely helpful as radio- (or chemo-) sensitizers. As stated in my Impact Statement for the grant.

"Our research has impact on two aspects of CaP that is refractory to XRT. First, it could enable clinicians to identify patients who would be good candidates for successful XRT, based on the initial biopsy, and thus greatly help in making appropriate clinical decisions. Second, our efforts to identify compounds that decrease the interaction of TLK1B with Rad9 could yield novel pharmacological approaches to radiosensitize even those patients whose cancers are not expected to respond well to XRT. The importance of finding such compounds cannot be overstated in relation to the benefit and the impact that they can have in the treatment of organ-confined CaP, and perhaps even in the case of bone metastases, where XRT is used to slow the growth of the lesions and for pain management. "



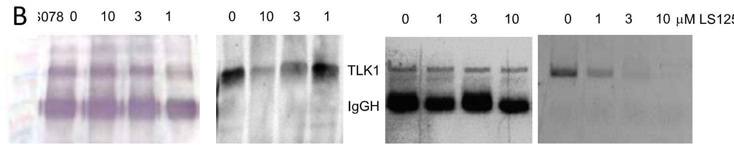
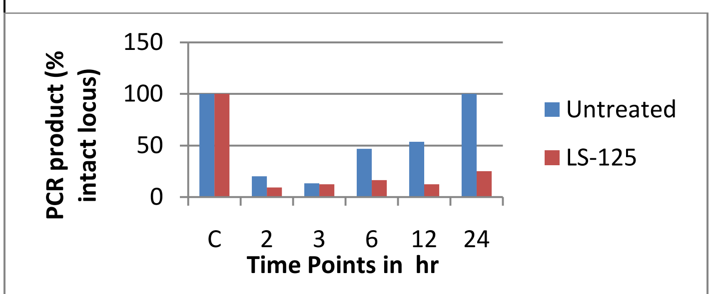


Fig. 1. Inhibitors of TLK1/1B. (**A**) Inhibitory curves of the 3 compounds in vitro using TLK1B and Rad9 peptide – the cpm are expressed relative to no inhibitors. (**B**) Inhibition of TLK1 from cultures. 293T cells were incubated for 1h with 2 the inhibitors. Extracts were prepared for IPs for TLK1; followed by auto-phosphorylation with γ^{32}P-ATP. WBs and autorads are shown.



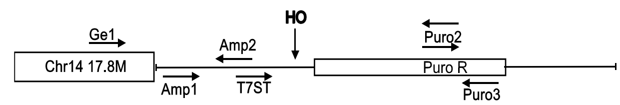


Figure 2: Time course Post Infection (in hr) with adeno-HO. Cells were treated or not for 12h with LS-125, before Ad-HO infection. Presence of the DSB and repair was followed by qPCR with primers T7 and Puro2. Diagram of the HO cassette integrated at chromosome 14 is shown above. Data points were normalized for the FABP gene as described in (10).

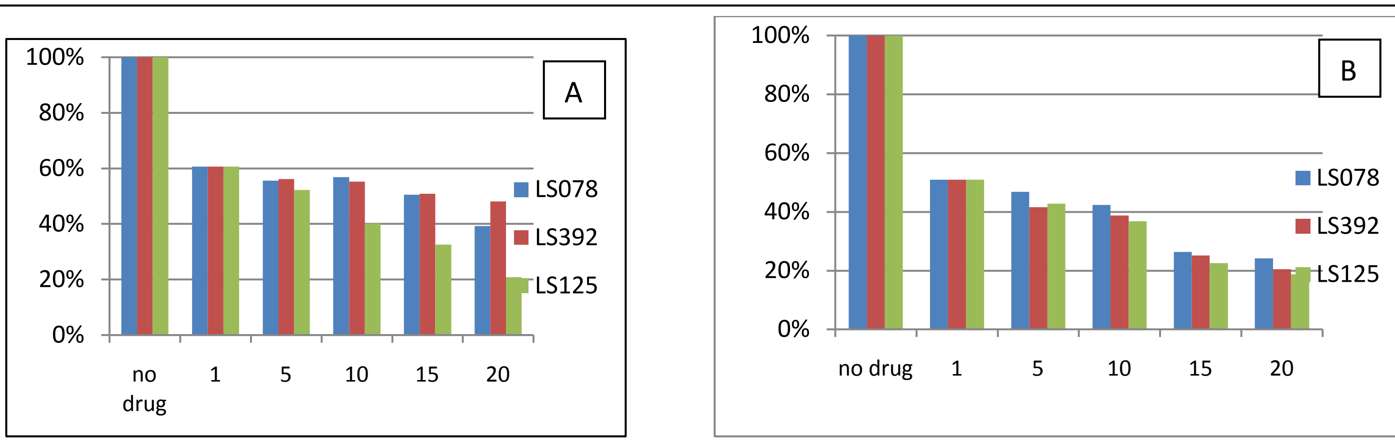


Fig. 3: Cell viability of PC-3 (A) or FDU-145 (B). C ells were incubated for 24h with three TLK inhibitors compared to doxorubicin alone, and viability was determined with CellTiter96 assay. Addition of doxorubicin alone (5 μM) was taken as 100% (no TLK drug), but represents ~40% killing. Viability was also monitored microscopically, and presence of apoptotic cells was noted.

The inhibitors worked as expected on the pattern of phosphorylation of Rad9-S328. After treatment of PC-3 cells with H2O2 to generate breaks, there is a corresponding wave of phosphorylation and dephosphorylation of S328 after completion of repair (A) that matches the pattern of activity of TLK1/1B. Generation of DSBs causes transient inhibition of TLK1 and simultaneous synthesis of TLK1B, which then results in hyper-phosphorylation of S328 upon restoration of kinase activity [Fig. 4 (2)]. Once H202 is degraded and DSBs and SSBs are repaired (4-8h), the phosphorylation returns to baseline. The inhibitors, LS125 in particular, blocked the activation of TLK and consequent phosphorylation of Rad9(S328) (Fig. 4).

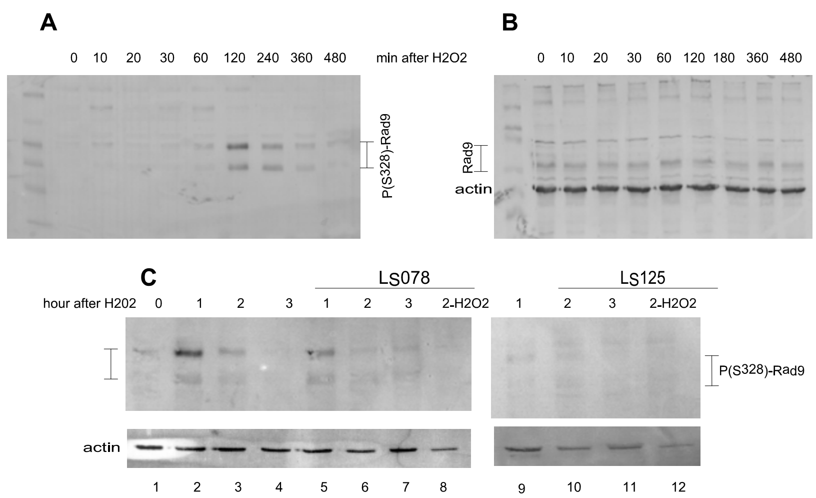


Figure 4. Pattern of phosphorylation of Rad9 (S328P) upon addition of H2O2 (lanes 1-4), and blockage by the TLK inhibitors in PC3 cells. Note that Rad9 appears as two main bands with either a pan-reactive anti-Rad9 (B), as previously shown (10), or with the phospho-specific Ab (A, C) (2). Samples incubated with drugs for 2h, with no H2O2 are shown in lanes 8 and 12.

REPORTABLE OUTCOMES

Papers published:

1. Kanikarla-Marie, P, Ronald, S, and De Benedetti, A (2011) Nucleosome resection at a double-strand break during Non-Homologous Ends Joining in mammalian cells – implications from repressive chromatin organization and the role of ARTEMIS. BMC Research Notes 4:13.
2. Ronald, S, Sunavala-Dossabhoy, G, Adams, L, Williams B, and De Benedetti, A. (2011) The Expression of Tousled Kinases in CaP Cell Lines and its Relation to Radiation Response and DSB Repair. The Prostate. Feb 14, Epub.
3. Palaniyandi, S, Odaka, Y, Green, W, Abreo, F, Caldito, G, De Benedetti, A, Sunavala-Dossabhoy, G (2011) Adenoviral Delivery of Tousled Kinase for the Protection Salivary Glands against Ionizing Radiation Damage. Gene Therapy 18(3):275-282.
4. De Benedetti A. (2010) Tousled kinase TLK1B mediates chromatin assembly in conjunction with Asf1 regardless of its kinase activity. BMC Research Notes 3:68.

Grants applied for:

De Benedetti, PI. NIH. TLK1 in DSB repair. 07/01/2012 -06/30/2016 (total cost $ 1,293,360)

Current personnel under training from this award: Abhijit Rath, graduate student; Sanket Awate, graduate student; Sharon Ronald, research associate.

References (recent papers published by other groups that are confirmatory of our work).

Silencing of **Tousled**-like kinase 1 sensitizes cholangiocarcinoma cells to cisplatin-induced apoptosis.
Takayama Y, Kokuryo T, Yokoyama Y, Ito S, Nagino M, Hamaguchi M, Senga T.
Cancer Lett. 2010 Oct 1;296(1):27-34. Epub 2010 Apr 9.
Phosphorylation-mediated control of histone chaperone ASF1 levels by **Tousled**-like kinases.
Pilyugin M, Demmers J, Verrijzer CP, Karch F, Moshkin YM. PLoS One. 2009 Dec 16;4(12):e8328.
The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non small cell lung carcinoma cells to DNA double-strand break induced cell death.
Polischouk AG, Holgersson A, Zong D, Stenerlöw B, Karlsson HL, Möller L, Viktorsson K, Lewensohn R. Mol Cancer Ther. 2007 Aug;6(8):2303-9.

The Expression of Tousled Kinases in CaP Cell Lines and Its Relation to Radiation Response and DSB Repair

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BACKGROUND. The *T*ousled-*l*ike *k*inases (TLKs) function in processes of chromatin assembly, including replication, transcription, repair, and chromosome segregation. TLK1/1B interacts specifically with the chromatin assembly factor Asf1, a histone H3-H4 chaperone, and with Rad9, a protein involved in DNA repair, and these interactions are believed to be responsible for the action of TLKs in double-strand break repair and radioprotection.
METHODS. Western blotting and RT-PCR were used to analyze the expression of TLK1, TLK1B, and TLK2 in a panel of prostate cancer (CaP) cell lines. The pattern of radiotolerance in the cell lines was analyzed in parallel. DU145 and PC-3 cells were also probed with assays utilizing transfected plasmids that could be cleaved in vivo with adeno-expressed HO nuclease to assess the potential contribution of TLK1/1B in DSB repair.
RESULTS. This is the first report of TLKs' expression in a panel of CaP cell lines and their relationship to radioresistance. Furthermore, expression of TLK1B in non-expressing PC-3 cells rendered them highly resistant to radiation, and conversely, knockdown to TLK1/1B in expressing DU145 reduced their radiotolerance.
CONCLUSIONS. TLKs appear to be intimately linked to the pattern of resistance to DNA damage, and specifically DSBs, a finding that was not reported before for any cell lines, and certainly not systematically for human prostate cell lines. *Prostate*

KEY WORDS: **TLKs expression; CaP cell lines; radioresistance**

INTRODUCTION

A high percentage of human tumors, including cancer of the prostate (CaP), show mutations in DNA repair genes and checkpoint functions that make them overly dependent on alternative pathways for survival. Unfortunately, this can result in carcinomas that are highly resistant to radiation therapy (XRT) from failsafe repair mechanisms.

Whereas there have been significant improvements in the technology and delivery of XRT for prostate cancer, making it a good alternative to surgery, many men are not cured by this therapy and ultimately die from progressive disease. A systematic review of nearly 500 randomized controlled trials and observational studies suggests that despite changes in XRT delivery, small differences in outcome have been achieved and a significant number of men still fail XRT [1]. We believe that one underlying reason for this failure is that at a molecular level, the surviving prostate cancer cells have adopted a highly efficient DNA repair mechanism that renders them insensitive to killing by XRT.

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The authors declare no conflict of interests with any part of this work.
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In the past few years significant evidence has accumulated that the *T*ousled-*l*ike *k*inases (TLKs) are involved in DNA damage [2] and promote repair of double strand breaks (DSBs) [3], and we have significantly advanced the knowledge of its mechanism of action [3,4–6]. In breast cancer, elevated expression of TLK1B corresponds to poor response to XRT and doxorubicin [7,8]. We postulated that its expression could serve as a marker for prognosis as well as a target for therapeutic intervention. In preliminary studies, we recently found that TLK1B is elevated in a number of radical prostatectomy specimens and we are expanding these studies to include tissues from men who have failed XRT and subsequently undergone salvage prostatectomy. More importantly, there are no studies that correlate directly the expression of TLK1, or its splice variant TLK1B, to the pattern of radiation sensitivity of either breast or CaP cell lines. In fact, there is only one study that relates knockdown of TLK1 in cholangiocarcinoma cells to sensitization to cisplatin [9]. The experiments presented here link the expression of TLK1 and TLK1B (which we often refer to as TLK1/1B) to radiosensitivity in CaP cell lines.

The TLKs are involved in chromatin assembly, DNA repair, transcription, and chromosome segregation ([3] and references therein). Evidence also exists about a link between TLKs and a DNA damage relay, since activity of TLK1 is inhibited by IR and genotoxins. The inhibition is mediated by ATM via Chk1 by direct phosphorylation at S695 [2]. These findings suggested a functional cooperation between ATM and Chk1 in propagation of a checkpoint response mediated by transient inhibition of TLK1, which may regulate processes involved in chromatin assembly [2]. However, the kinase activity of TLK1/1B is only one activity of these proteins, which are also chaperones for their substrates even in the absence of kinase function [10]. TLK1/1B interacts specifically with the chromatin assembly factor Asf1 [10–12] and Rad9 [3], and we have presented evidence that TLK1B promotes repair by processing of the DSB ends and disassembly of chromatin near the DSB to facilitate recruitment of repair proteins [3,6]. Since Rad9 is a critical mediator in the response to DNA damage and in repair [13] and specifically of DSBs [14], it seemed that the TLK1-Rad9 interaction would be very important in implementing the mechanism of TLK1B-mediated radioprotection. In fact, the effect of TLK1B on survival from DSB-mediated cell killing must be mediated by its activity on Rad9, since overexpression of TLK1B in Rad9−/− ES cells could not restore resistance to radiation in the absence of Rad9 co-expression [3]. It is then logical to assume that this interaction of TLK1B and Rad9 is important for enacting this correlation between elevated TLK1B and refractory cancers [8]. While we were working out the molecular mechanisms of radioprotection by TLK1B and Rad9, three studies were published which portend the high significance of Rad9 in CaP prognosis and progression. In one, Rad9 was associated with tumor stage and was reported to regulate tumor growth in mice [15]. In another, the investigators found that Rad9 contains androgen-responsive elements and that its expression is also androgen-regulated [16]. In a third study, Rad9 acted as a co-repressor of AR transactivation [17]—all of which suggested that Rad9 expression may be a significant part of the ''androgen switch'' that leads to cancer cell survival, and that Rad9 has functions beyond DNA repair that make it clinically relevant as a biomarker or in tumor growth control. Correspondingly, expression of TLKs (with or without Rad9) may be a significant marker of radioresistance in CaP cell lines and likely in cancerous samples.

RESULTS AND DISCUSSION

We now present the first report on the expression of TLK1, TLK1B, and TLK2 in prostate cancer cell lines. TLK1 was expressed in all cell lines (Fig. 1A) including the immortalized non-cancer RWPE-1 cells. A notable exception, however, was the PC-3 cell line and the derived metastatic line PC-3M, which did not express TLK1 or the TLK1B splice variant. This was initially puzzling since *Tousled* is held to be essential [18]. However, it later became apparent that these cells express TLK2 (Fig. 1C), the second *Tousled* gene in humans [19], which can presumably compensate for the lack of TLK1/1B expression. Notably, TLK2 was not expressed in the other CaP lines, but was expressed in RWPE-1 (Fig. 1C). The expression of TLK1 was rather uniform in the various cell lines (panel A), whereas that of TLK1B was more variable. Since the expression of TLK1B is also controlled at the level of translation by eIF4E [5,20], we decided to probe the expression of TLK1 and TLK1B at the mRNA level (Fig. 1B). In general, the pattern of expression of the transcripts matched that seen on the WB for both splice variants, but leaving room for the fact that the RT-PCR was mainly qualitative, there were a few points of notice. For example, the TLK1B mRNA levels in LNCaP and MDA-PCa2b were very similar, but there was more protein in MDA-PCa2b, possibly an indication of translational regulation. The most obvious result was that PC-3 and PC-3M did not show any TLK1/1B transcripts, while RWPE-1 did not express TLK1B. Establishing the reasons for the lack of TLK1 expression in the PC-3 cells was beyond the goals of our work. However, we briefly approached the possibility that the gene was deleted or rearranged. Hence, we amplified two gene segments in Exon1 and Exon3. In this case, we

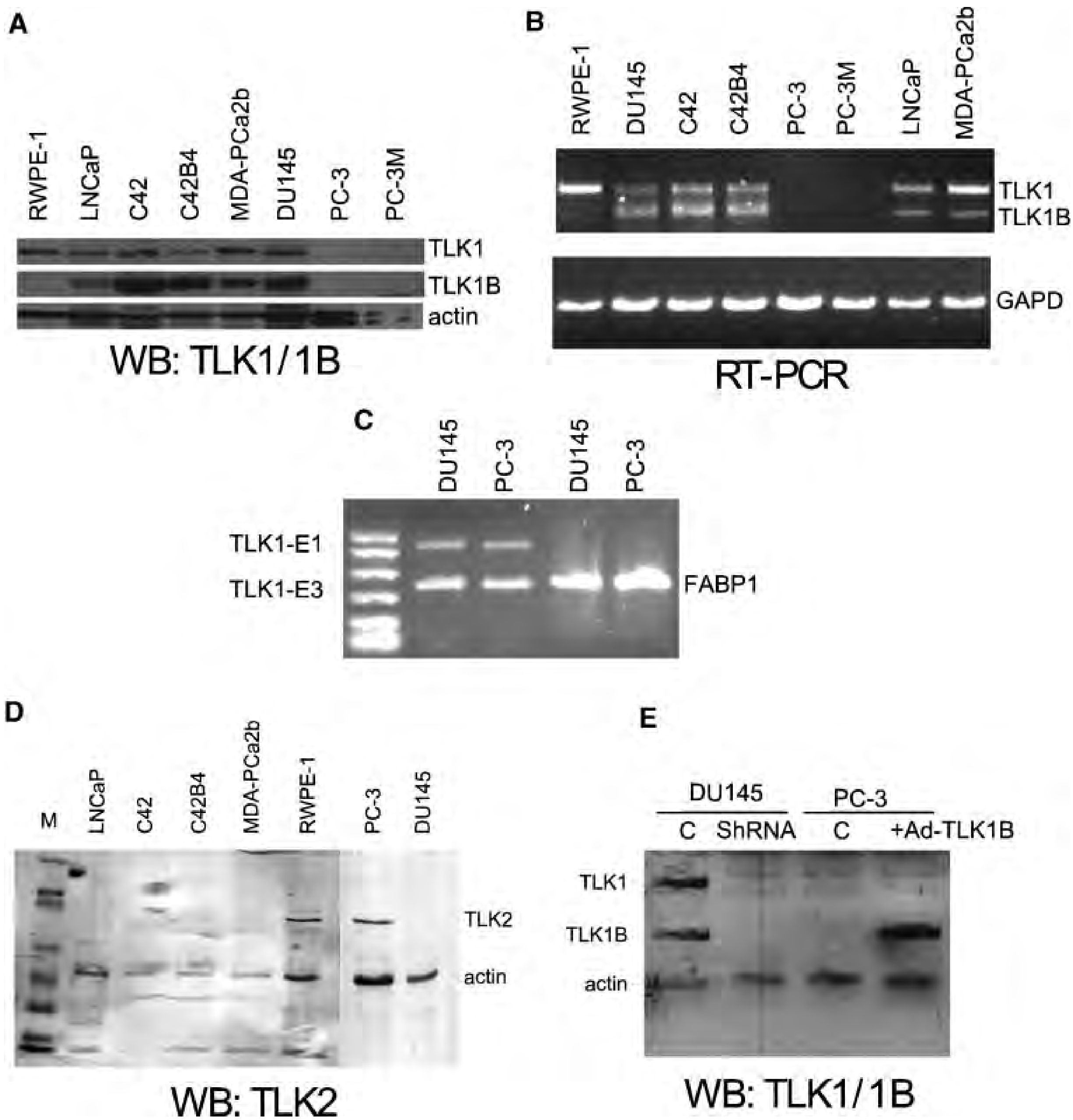

Fig. 1. **A**: Western blot of TLK1 and TLK1B in the indicated panel of CaP cell lines. The blot for TLK1 was reacted with an antibody from Cell Signaling that reacts specifically with the N-terminus of TLK1 and hence, not TLK1B. The TLK1B blot was reacted with our lab rabbit antiserum that recognizes both proteins. This antibody has been extensively tested and reacts only with TLK1 variants; and was originally described in Ref. 5. **B**: Expression of TLK1 and TLK1B mRNA by RT-PCR. Primer sequences and cycling conditions are available upon request. **C**: Genomic PCR for TLK1. Two segments of DNA contained in Exon 1 and Exon 3 of TLK1 were amplified from genomic DNA isolated from DU145 and PC-3 cells. **D**: Western blot of TLK2 using a rabbit antibody from Bethyl Laboratories. **E**: Western blot of TLK1/1B. DU145 were transfected for 24 hr with either of two shRNAs from Origene (cat# TI37902) that reacts with both TLK1 and TLK1B. PC-3 cells were infected for 4 hr with Ad-TLK1B (100 MOI) and cell lysates were probed for TLK1B expression.

really did not need a quantitative result since all we wanted is a yes or no answer for presence of the gene. Nonetheless, the two products seem to be in exactly the same amount in DU145 and PC3, and they seem to be in the same general amplification range of FABP1, which should be a single copy gene also on chromosome 2. This suggests that the TLK1 gene (on Chr 2) is present in PC-3, and short of having to sequence the entire gene, we suspect that it is probably all there but not expressed.

Our goal was to correlate the pattern of TLK1/1B expression with radiosensitivity of the different cell lines. While there are a number of contradictory studies, LNCaP and PC-3 are generally considered to be radiation sensitive, while other lines are more resistant. While this turned out to be true also in our study (see Fig. 2), we first wanted to test the likelihood of a more direct involvement of TLK1/1B in the radiation response. Hence, DU145, which strongly express TLK1 and TLK1B, were treated with different shRNAs to knock down the expression of both proteins (Fig. 1D). Both proteins were suppressed >90% and notably the cells remained viable during the 2 days of the experiment, which made it possible to carry out radiation survival curves. There was no evidence of a compensatory expression of TLK2 in these cells 2 days after transfection with the shRNA (not shown). DU145 were the most resistant cells in our panel of lines (Fig. 2A), but knockdown of TLK1/1B greatly sensitized DU145 to radiation (nearly one log at 6 Gy). We used as a non-target control shRNA for GFP, which gave the same pattern of resistance as non-transfected DU145 (Fig. 2A). Hence, knockdown of TLK1B/1B rendered DU145 much more sensitive to killing by radiation. However, we must caution that the radiosensitization may not be directly related to the role of these proteins in DNA repair, but could result instead from an effect on cell cycle progression, which has consequences on the DNA damage response and repair. In fact, knockdown of TLKs can result in an S phase arrest [21]. Since radiation causes alterations in cell cycle by itself it would be very difficult to separate the effect of TLK1 knockdown with that of radiation.

To complement this experiment, we infected PC-3 cells that do not express TLK1/1B with Ad-TLK1B and determined its expression by Western blot (Fig. 1D). A significant amount of TLK1B was made in these cells, and migrated at the same approximate position as the protein expressed in DU145. Whereas PC-3 cells are very sensitive to radiation, after infection with Ad-TLK1B, the cells became almost completely resistant, all the way to 8 Gy (Fig. 2B). Cells infected with control Ad-GFP showed the same pattern of high radiosensitivity as non-infected PC-3 (not shown). A field of the cells infected with Ad-GFP is shown in panel C, which demonstrated that most of the cells in culture were infected. We should note that the cells infected with Ad-TLK1B (and of course those infected with Ad-GFP) continued to divide normally during the 3 days of the experiment and did not show any adverse effects or evidence of cell cycle alterations; in this case we are more confident that the increased radiotolerance was not due to cell cycle arrest. In fact, we have recently published the use of the Ad-TLK1B virus for the protection of salivary glands from radiation treatment in rats [22]. Some of those experiments lasted 1 month, and there were no visible effect on the acinar salivary cells on histopathology, other than high expression of TLK1B [22].

The other cell lines in this study were variably sensitive to radiation (Fig. 2C), with RWPE-1 being more resistant, possibly due to the fact that these cells express both TLK1 and TLK2. These experiments present the first evidence that the expression of TLKs may correlate with radiosensitivity, and while we are aware that the phenomenon of resistance is complex and likely involves numerous proteins, this evidence is provocative.

The molecular mechanism of radioresistance by TLK1/1B is currently under investigation, and it clearly involves several facets of DNA repair, intrinsic and at the chromatin level. We previously developed an assay of DSB repair introduced with the yeast HO endonuclease expressed from adenovirus. For this assay, a genomic HO target site was created in MM3MG cells (mm3-HO) [3] that can be probed by PCR with primers flanking the cut site. Before cleavage, a PCR product is obtained, whereas after cleavage the amplicon is lost since the cut site resides between the primers (see diagram published in Ref. 3). A derivative cell line was also created that expresses TLK1B constitutively. We have now repeated this assay of cleavage and repair during a time course of infection, but in this case we have used only mm3-HO cells, with and without simultaneous co-infection with Ad-TLK1B (Fig. 2D). During a time course of infection (200 MOI), there is loss of PCR signal, as previously published. For control cells, repair begins at about 3 hr post-infection (PI) and recovers completely ~24 hr, whereas for cells co-infected with Ad-TLK1B repair becomes evident by 1 hr and is complete ~12 hr PI. This is a direct demonstration that one effect of TLK1B is to hasten DSB repair.

We are currently developing new systems based on the Ad-HO nuclease that will allow study of repair in most cells without having to generate integrated clonal derivatives. One such system is based on a plasmid reporter expressing GFP and Luc, each driven by a CMV promoter (Fig. 3A). The continuous production of Luc, which turns over rapidly, is disrupted after HO cleavage. Likewise, the expression of HO, driven by the adeno early-control region, is only of brief duration [3] allowing to study repair in the absence of continuous cleavage of the target site by the HO nuclease. PC-3 and DU145 cells were transfected with the reporter plasmid; 12 hr later, a time when we know expression of GFP and Luc reached its maximum, the cells were infected with Ad-HO (Fig. 3). In un-infected cells, Luc expression remained constant for the next 2 days at around 50,000 RLU. In contrast, 12 hr after infection,

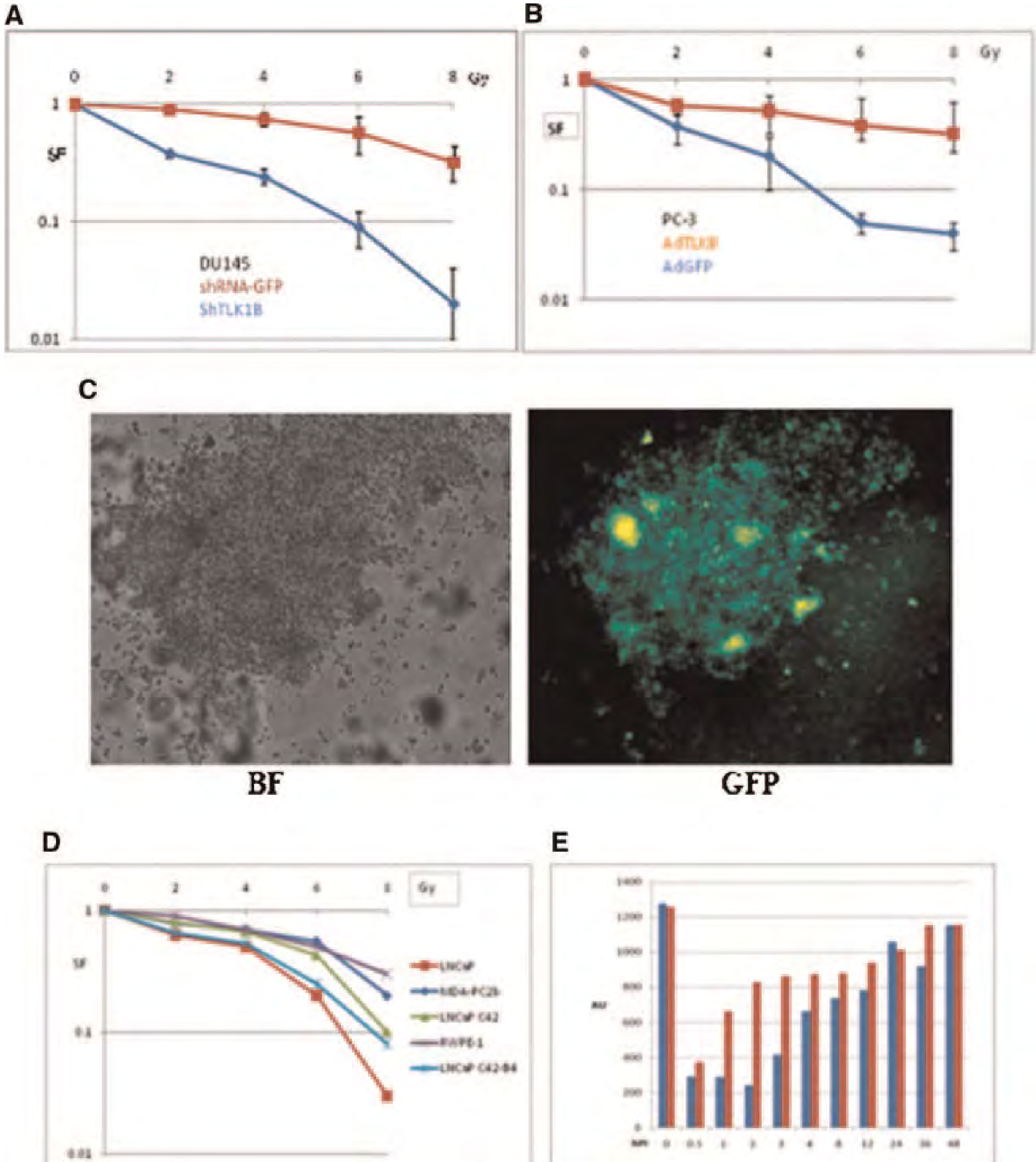

Fig. 2. **A**: Pattern of radiation response in DU145 after transfection with shRNA against TLK1/1B or non-target (GFP)-shRNA) for 24 hr. **B**: Pattern of radiation response in PC-3 cells before and after infection with Ad-TLK1B or control Ad-GFP. **C**: Visual estimate of the efficiency of infection of PC-3 cells with Ad-GFP. **D**: Pattern of radiation response in different CaP lines. The irradiation was carried out with Cs irradiator in tubes containing 10^5 cells/ml, which were then immediately plated in 96-well plates (10^4 cells) and monitored for viability the next day with CellTiter 96 reagent (Promega). At least two separate experiments were carried out in triplicates and the median of each data point is plotted with SD (error bars). The X-axis is Gy and SF is surviving fraction. **E**: Time course in hours post-infection with Ad-HO resulting in cleavage and repair at the genomic HO site in mm3-HO cells. The obtained by PCR across the DSB were normalized for FABP, a single copy gene on mouse chromosome 6, as described in Ref. 3. The bands were quantified with ImageJ and reported as AU.

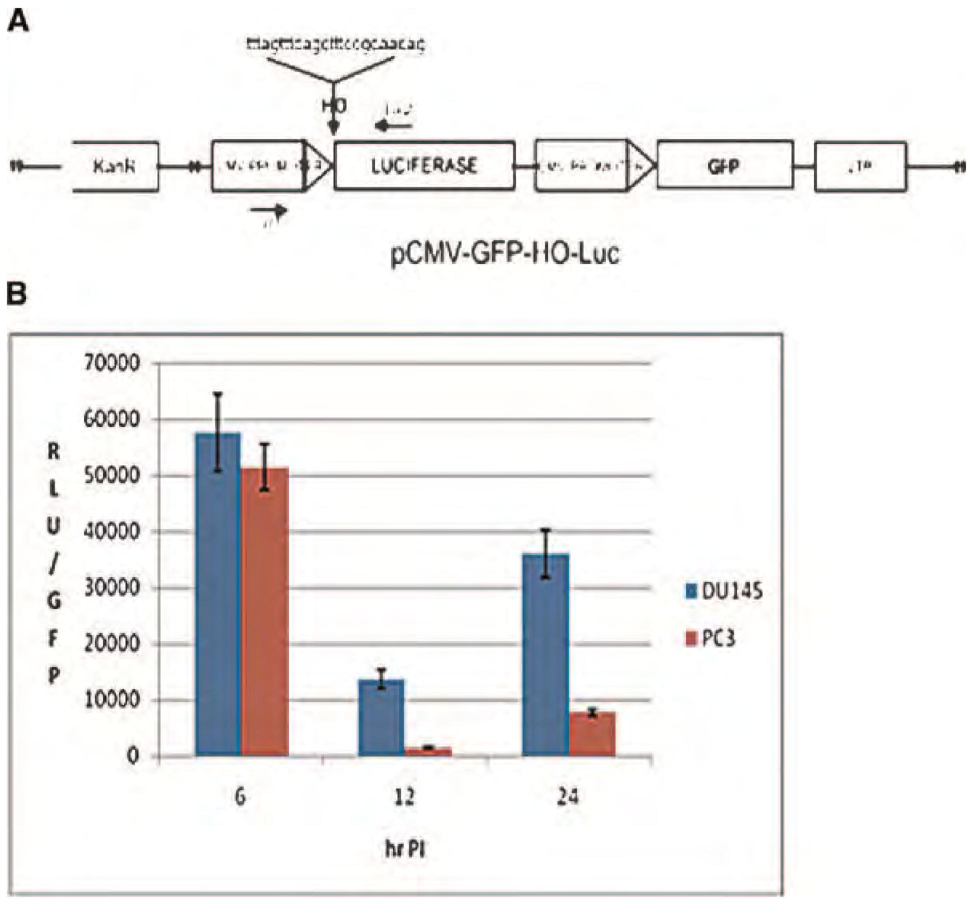

Fig. 3. **A**: Sketch of the plasmid used for the transfections. **B**: DU145 and PC-3 cells (10^4) were transfected with the reporter plasmid, and 12 hr later infected with Ad-HO. Time points were taken as indicated, and Luc results of triplicates are shown after normalization for GFP fluorescence.

RLU were reduced fivefold. However, at 24 hr, a time when we know maximal plasmid repair has occurred as measured by PCR (e.g., Fig. 2D), 63% of Luc activity was recovered in DU145, but only 15% in PC-3. Hence, with this system it has become possible to directly relate the rate of DSB repair at a single engineered site with the pattern of radiation sensitivity (or other agent that causes DSBs). This experiment clearly indicated that PC-3 cells are inferior to DU145 cells in repair activity, and while we could not demonstrate that the difference is directly linked to presence of TLK1B/1B, this is quite possible. To prove this directly, we are currently generating in vitro repair systems from PC-3 and DU145 extracts, similarly to what we have recently published for MM3MG cells [6] that showed a dependence on TLK1/1B, either by exogenous supplementation or immunodepletion. We have considered testing the same system with DU145 transfected with shRNA against TLK1/1B, but this presents technical problems. Transfecting shRNA and then also the plasmid reporter gives very complicated results that require many more controls, and still leave some uncertainty. Similarly, infecting PC-3 first with Ad-TLK1B and Ad-HO, and later on with the reporter plasmid presents several technical concerns. Thus, we have only used the natural expression pattern of PC-3 and DU145 to do these experiments, which was the primary goal of our study in any case.

CONCLUSIONS

To date, no reliable markers have been identified that can guide the oncologists to the best initial treatment, and despite the fact that most men today are being diagnosed with a sufficiently early, organ-confined CaP, a significant proportion will fail any of the treatment options. In general, advanced stage and grade are associated with treatment failure, but at the root of these failures are molecular changes that are not appreciated at the time of treatment. External beam radiation therapy has developed technologically over the last several years and is now used alone or in combination with surgery or hormone therapy. However, even with improvements in technology or delivery strategies, many men still fail XRT. Success for XRT for localized CaP is largely based on the assumption that dividing cancer cells are preferentially killed with respect to non-dividing, normal cells. There are problems with this oversimplification. For one, CaP is a slow-dividing cancer, while nearby cells in the gut are rapidly dividing and susceptible to XRT damage, resulting in serious complications. Perhaps most importantly, for some patients, their cancer cells may be resistant to XRT because of proficient DNA repair, which could result in incomplete killing of the primary tumor cells. Rather than changing the dosage or frequency of XRT, changes to the cells themselves to make them more sensitive to killing will improve outcome. We showed in this study that the expression of TLK1/1B (and perhaps TLK2) correlates with radioresistance in a panel of CaP cell lines. To further support TLK1/1B's role in radioresistance, TLK1/1B knockdown rendered DU145 cells more radiosensitive, while overexpression of TLK1B in non-expressing PC-3 cells rendered them very resistant. These results should propel studies on the expression of TLKs in CaP specimens and their relationship to XRT response. To that end, we are presently conducting a more definitive assessment using tissues from salvage prostatectomy from men who have failed XRT in order to determine the association between TLK1B expression and risk of XRT failure.

ACKNOWLEDGMENTS

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BMC Research Notes

SHORT REPORT Open Access

Nucleosome resection at a double-strand break during Non-Homologous Ends Joining in mammalian cells - implications from repressive chromatin organization and the role of ARTEMIS

Preeti Kanikarla-Marie, Sharon Ronald, Arrigo De Benedetti*

Abstract

Background: The *S. cerevisiae* mating type switch model of double-strand break (DSB) repair, utilizing the HO endonuclease, is one of the best studied systems for both Homologous Recombination Repair (HRR) and direct ends-joining repair (Non-Homologous Ends Joining - NHEJ). We have recently transposed that system to a mammalian cell culture model taking advantage of an adenovirus expressing HO and an integrated genomic target. This made it possible to compare directly the mechanism of repair between yeast and mammalian cells for the same type of induced DSB. Studies of DSB repair have emphasized commonality of features, proteins and machineries between organisms, and differences when conservation is not found. Two proteins that stand out that differ between yeast and mammalian cells are DNA-PK, a protein kinase that is activated by the presence of DSBs, and Artemis, a nuclease whose activity is modulated by DNA-PK and ATM. In this report we describe how these two proteins may be involved in a specific pattern of ends-processing at the DSB, particularly in the context of heterochromatin.

Findings: We previously published that the repair of the HO-induced DSB was generally accurate and occurred by simple rejoining of the cohesive 3'-overhangs generated by HO. During continuous passage of those cells in the absence of puromycin selection, the locus appears to have become more heterochromatic and silenced by displaying several features. 1) The site had become less accessible to cleavage by the HO endonuclease; 2) the expression of the puro mRNA, which confers resistance to puromycin, had become reduced; 3) occupancy of nucleosomes at the site (ChIP for histone H3) was increased, an indicator for more condensed chromatin. After reselection of these cells by addition of puromycin, many of these features were reversed. However, even the reselected cells were not identical in the pattern of cleavage and repair as the cells when originally created. Specifically, the pattern of repair revealed discrete deletions at the DSB that indicated unit losses of nucleosomes (or other protein complexes) before religation, represented by a ladder of PCR products reminiscent of an internucleosomal cleavage that is typically observed during apoptosis. This pattern of cleavage suggested to us that perhaps, Artemis, a protein that is believed to generate the internucleosomal fragments during apoptosis and in DSB repair, was involved in that specific pattern of ends-processing. Preliminary evidence indicates that this may be the case, since knock-down of Artemis with siRNA eliminated the laddering pattern and revealed instead an extensive exonucleolytic processing of the ends before religation.

Conclusions: e have generated a system in mammalian cells where the absence of positive selection resulted in chromatin remodeling at the target locus that recapitulates many of the features of the mating-type switching system in yeast. Specifically, just as for yeast HML and HMR, the locus had become transcriptionally repressed; accessibility to cleavage by the HO endonuclease was reduced; and processing of the ends was drastically

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changed. The switch was from high-fidelity religation of the cohesive ends, to a pattern of release of internucleosomal fragments, perhaps in search of micro-homology stretches for ligation. This is consistent with reports that the involvement of ATM, DNA-PK and Artemis in DSB repair is largely focused to heterochromatic regions, and not required for the majority of IR-induced DSB repair foci in euchromatin.

Background

Double Strand Breaks (DSBs) are the most serious form of DNA damage, yet they occur frequently by generation of reactive oxygen species during normal metabolism or by external agents such as ionizing radiation (IR). Furthermore, specific DSBs are genetically programmed, as in immunoglobulin gene rearrangements or during mating type switching in the yeast *S.cerevisiae*. DSBs are generally dealt with by homologous recombination repair (HRR) or non-homologous end-joining (NHEJ). Perhaps the best studied model of DSB repair is the mating type switching of *S. cerevisiae*, which requires the coordinated action of many proteins, and minimally consists of the HO endonuclease and a host of components required for a gene conversion event that utilizes the silent mating cassette of the opposite type as the donor (for a review, see [1]). Following specific cleavage at the MAT locus by the HO endonuclease, the recessed strand is further processed by an endonuclease and/or exonuclease to generate long stretches of ssDNA capable of strand invasion and then as acceptors for HR replication/repair utilizing the information from HML or HMR as the template. The precise involvement of the nuclease in the resection process is unclear, but Sae2 has been implicated [2]. However, clearly the process is complex and highly regulated since, for example, strand resection is suppressed in a strain deleted for the HML and HMR [1]. In that case, repair occurs only via NHEJ and with minimal processing of the ends, which are then capped by yKu and the DSB-binding complex Mre11/Rad50/Xrs2p. The silent cassettes (HML and HMR) are characteristic heterochromatic regions that are established by several factors, and minimally by the four Sir proteins, Rap1 and ORC, and resulting in repressive chromatin. This includes suppression of transcription of genes present at or near HML and HMR, and inaccessibility to cleavage by the endogenous HO nuclease at HML and HMR. In fact, the activity of HO is strongly influenced by the chromatin state of its target sequence.

In mammalian cells, NHEJ is the predominant pathway of DSB repair, and although NHEJ-defective cell lines show marked defects in DSB repair and sensitivity to IR, cells defective in ATM, a key protein kinase involved in repair and cell cycle arrest, repair most DSBs normally [3]. Thus, NHEJ can function in the absence of ATM signaling. However, 10-20% of visible IR-induced DSBs (repair foci) are repaired with slow kinetics and appear to require ATM and a target nuclease, Artemis, involved in ends processing [4]. Interestingly, these DSBs that persist in the presence of an ATM inhibitor localize to heterochromatic regions associated with markers of silenced chromatin, suggesting that ATM and Artemis are particularly important for repair of DSBs at condensed genomic regions [3]. Artemis is an ATM-dependent substrate after radiation [3]. Artemis is both an endouclease and exonuclease that has the capacity to cleave hairpin-ended DSBs and to remove 3'- or 5'-single-stranded overhangs, following binding of the DNA ends by the DNA-dependent protein kinase (DNA-PK) [4]. Furthermore, at least *in vitro*, the endouclease and exonuclease trimming are inversely regulated by DNA-PK, and depending on the type of ends (e.g., incompatible or blocked) [5]. Hence, the activity of PI3K-like kinases (PIKKs) can significantly affect the processing of DSB ends by Artemis, and particularly in the context of heterochromatic regions. Recent work has also indicated that Artemis is directly involved in generating the classic DNA fragmentation ladder obtained during apoptosis [6]. One hallmark of apoptosis is DNA degradation that first appears as high molecular weight fragments, followed by extensive internucleosomal fragmentation. DNA-PK is typically involved in the repair of DSBs by facilitating processing of damaged ends, but in the context of apoptotic conditions, it seems that DNA-PK and Artemis' roles shifts to that of final executioners [6].

We have recently described a system of DSB repair where we have transposed the minimal yeast HO endonuclease system to mammalian cells, by generating cells containing the HO target sequence and utilizing an HO-encoding adenovirus [7,8]. In this system, cleavage was very efficient (depending on the MOI), and repair occurred via simple ends-joining during a time course of infection - the HO enzyme is largely degraded at later times, thus avoiding continuous recutting of the site. While the repaired junctions were not isolated and re-analyzed from individual clones, the repair appeared to be predominantly a high fidelity religation of the 4-bp cohesive ends at the level of PCR-generated band. The HO target construct is integrated, and hence, we had not maintained the puromycin selection for many passages, as this did not appear necessary. We now report some interesting changes that occurred in these "unselected" cells that can be best explained by the establishment of heterochromatic, repressive chromatin at the locus.

Results and Discussion

We recently resurrected the Adeno-HO/MM3MG system to address one simple question: What would be the effect of adding wortmannin (WMN), a general inhibitor of PIKKs including ATM, ATR, and DNA-PK on the kinetics of repair of the single HO-mediated DSB? A diagram of the HO target cassette is shown in Figure 1A. The kinetics of cleavage and repair can simply be monitored as a loss and then recovery of the T7ST/Puro1 amplicon generated with primers flanking the HO cleavage site. Much to our dismay, very little cleavage was obtained at 200 MOI (Figure 1B), which was different than our previously published work [7,9]. In that work, cleavage was complete within 0.5-1 h post-infection (PI), and repair began variably after 3-10 h. We knew from other work ongoing in the lab that the virus stock was active, so that could not be a reason for the loss of cutting. Some cleavage was evident, but this did not appear to involve more than 50% of the cells. Where cleavage was detected, repair was complete by 8h PI. There appeared to be also a confusing smaller band of about 500 bp (asterisk), which we could not interpret at the time. This band was present only after inducing the DSB and it seemed to be a specific repair product. This product was less obvious at 36h PI, which corresponds to about 1.5-2 cell divisions later.

Since the HO nuclease is very sensitive to chromatin structure, we suspected that perhaps the site had become heterochromatic and hence partially inaccessible to cleavage. We then reapplied selection with puromycin (1 μg/ml). Nearly 80% of cells died, but this obviously did not involve general ejection of the construct, as this was clearly present by PCR. It seemed more likely that the locus had become transcriptionally inactive in the absence of selective pressure. The remaining 20% began dividing rapidly after a short lag and recovered fully in a few days. They were maintained under selection and named "reselected cells" (Figure 1B, C). We then repeated the infection/cleavage experiment with these cells, and also tested for the expression of the puro mRNA. Total RNA from unselected and reselected cells was isolated and first strand cDNA was obtained using olgo-dT, followed by semi-quantitative PCR with specific primers. As we had suspected, the expression of puro mRNA was significantly lower in the unselected cells in comparison to the reselected (Figure 1C) - the expression of Yes mRNA was used as a normalization control. The simplest explanation for these results is that the locus had become heterochromatic and partially silenced in the absence of selection, in at least a large fraction of the cells. We then carried out a time course of cleavage and repair in the reselected cells. This time cleavage was very efficient and essentially complete within ½ hr. Repair also began almost right away but did not reach the maximum until 4-8h later. The greater accessibility to HO nuclease could thus explain the more complete cleavage at the HO site, consistent with a possible decondensation of chromatin at the locus and increased puro expression following reselection. However, there was a second surprise. Apparently, only a fraction of cells (less than 50%) repaired the DSB with regeneration of the correct sized PCR product (labeled "puro" in Figure 1B). The remainder generated a short ladder of three smaller and discrete PCR products (Figure 1B - 4h longer run). Curiously, the spacing between these bands was highly reminiscent of a nucleosomal ladder with a separation of ~200 bp. However, the spacing between bands 2 and 3 is only 80-120 bp, suggesting that this segment might be occupied by a non-standard nucleosome or some other type of protein or protein complex. We will refer to that as "nucleoprotein complex 4". These PCR products were better separated on another gel in the right panel, and bands 1, 2 and 3 were excised and re-amplified. Upon close inspection, the PCR bands were not completely sharp, so they were shot-gun cloned in the *Sma*I site of Bluescript plasmid. Several clones obtained from each band were sequenced. Clones derived from each PCR band showed very high sequence identity of >98% along their coverage, with the main differences lying within the gaps at the breakpoints. The alignment of one representative clone from each band with the original HO-puro sequence is shown in Figure 2A. The gaps suggested spacing for the nucleosomes in the region, where the sequence flanking the T7ST primer and including the first nucleosome, to the left of the HO, was preserved. In contrast, progressively larger gaps on the right side of the HO site suggested removal of the next two nucleosomes and the following nucleoprotein complex 4. It is possible that the internucleosomal resection occurred beyond the boundaries of the T7ST and Puro1 amplicon in some cells; only such events would not have been picked up without using more distal primers. In summary, the specific pattern of cleavage and repair suggested that, unlike the precise regeneration of the segment at the HO joint obtained when the cells were first made, the reselected cells often repaired the DSB only after further resection, which apparently involved the removal of full nucleosomal segments. We should however caution that other explanations for this pattern of cleavage and religation are possible beside removal of nucleosomes. For example, there could be a specific pattern of DNA bending in that region that renders it locally exposed to discrete nucleolytic cuts. One distinct possibility is that the cuts

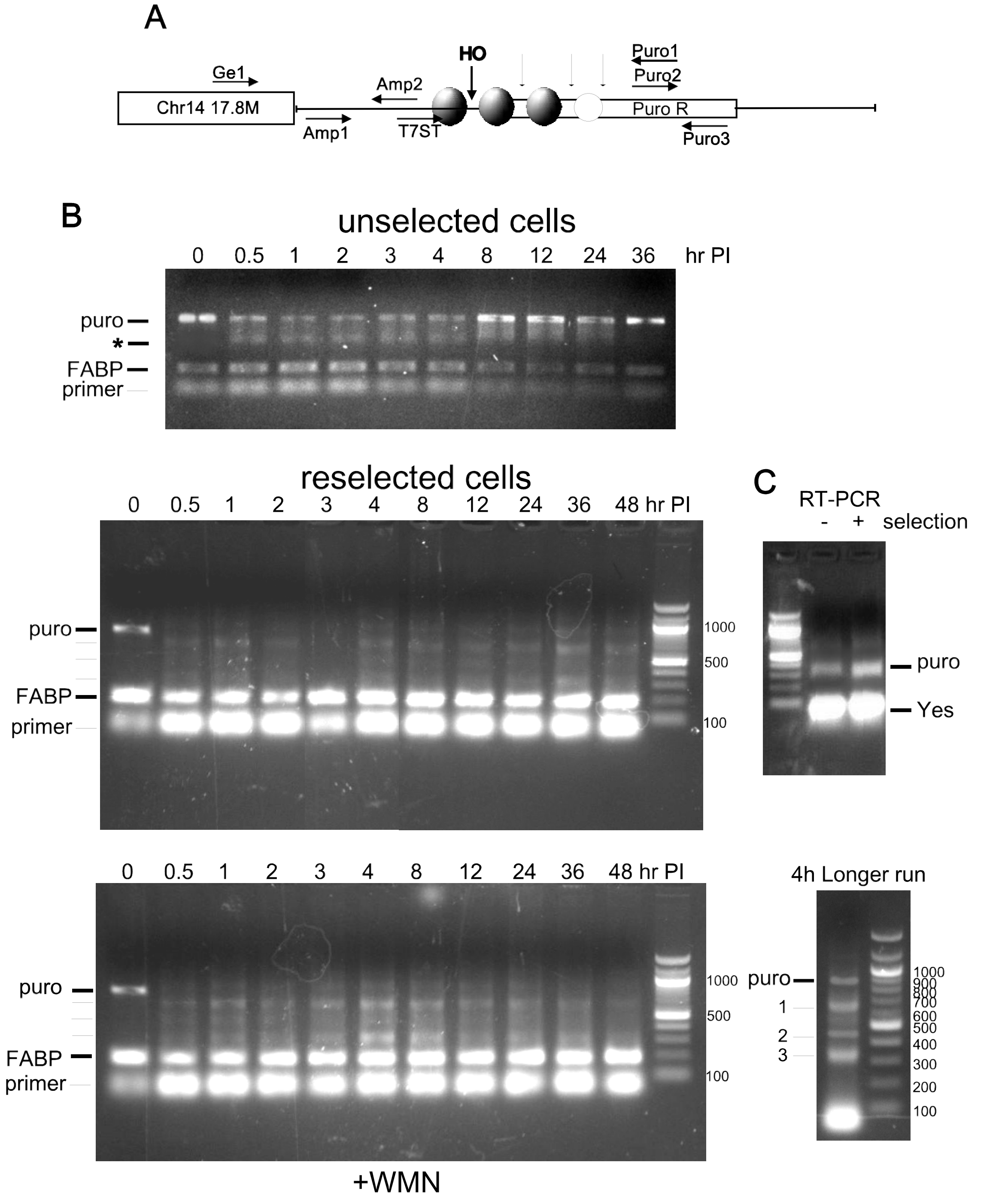

Figure 1 Pattern of cleavage and repair generated with Adeno-HO nuclease. A) Diagram of the integrated HO target cassette, including a representation of the HO cleavage site, position of the primers used, and putative positioning of nucleosomes and secondary cleavage sites. B) Kinetics of cleavage and repair (hours post-infection [PI] with adeno-HO) in unselected and puromycin reslected cells. For these experiments, 10,000 cells were infected at an MOI of 200 in 96-well plates, and the DNA was isolated at the indicated times with Wizard genomic DNA isolation kit (Pormega). The product of the original target (amplicon T7ST/Puro 1) is indicated for cells at the start of infection as “puro”. Other intermediate PCR bands are indicated by lines. FABP is a PCR product from a single-copy gene on mouse chromosome 6. Wortmannin (WMN; 30 μM) was added to the cells in the bottom panel throughout the time course post-infection. The products from 4h time point of the bottom panel were separated on a new gel to the right, using more material and a longer gel run. The two PCR reactions (Puro and FABP) were run in parallel with the same thermocycle settings and the products were mixed before loading each lane. C) RT-PCR for semi-quantitative analysis of the expression of puro mRNA. First-strand cDNA was produced with oligo-dT from 5 μg of total RNA from unselected and reselected cells. Puro mRNA and Yes mRNA were amplified in parallel for estimation of their relative expression.

do not occur within the linker segments, as we have drawn in Figure 2A, but rather are rotationally phased on the surface of the nucleosomes. In such case, the nucleosomes would still have to be spatially organized in the cell population but for instance, they may not be positioned where we drew them. In this respect, we should also stress that we do not claim that the ~500 bp PCR product obtained in Figure 1B with the unselected cells (top panel) is the same as bands 1 or 2 of the reselected cells (bottom panels). While this is

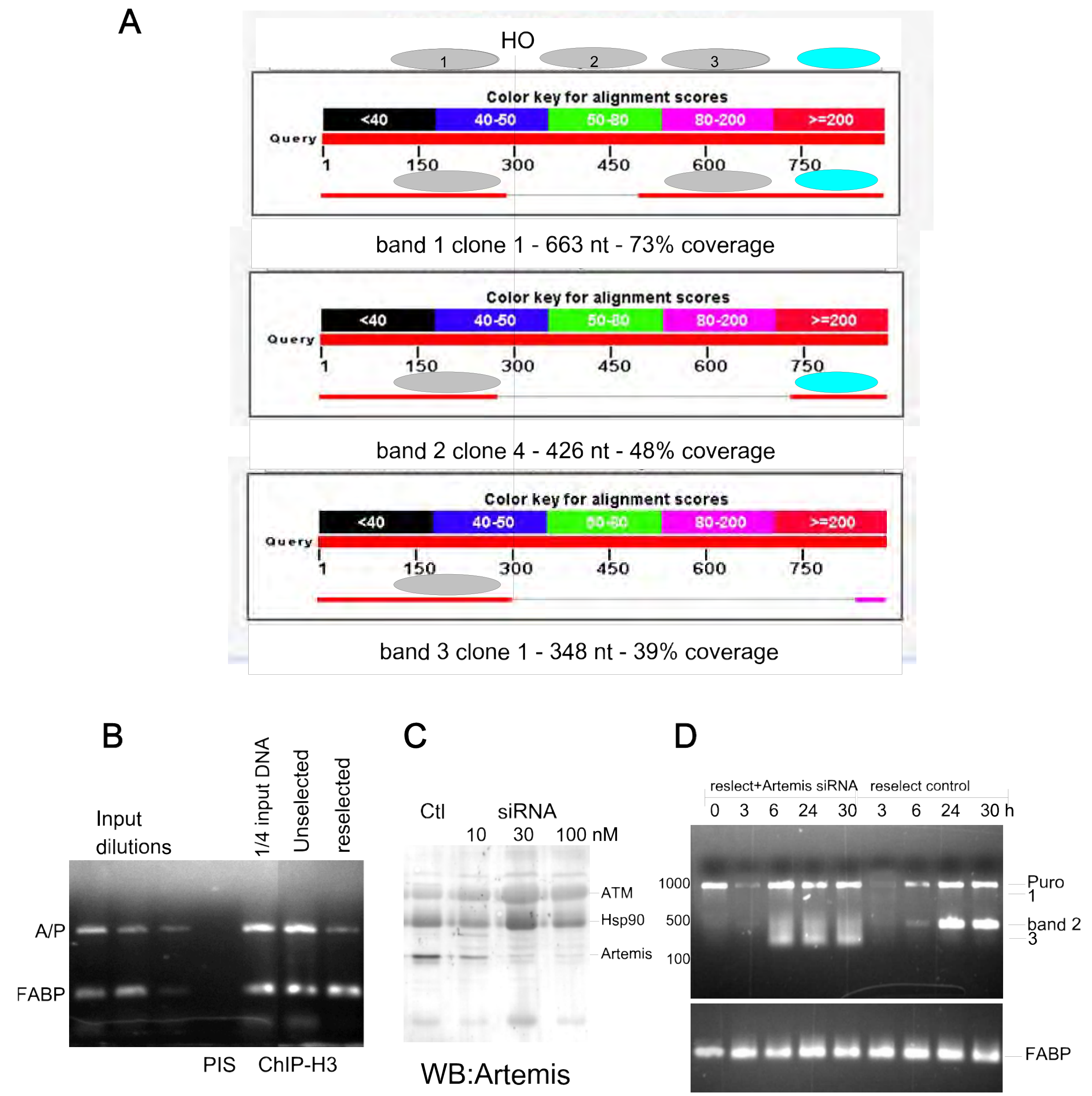

Figure 2 Extent of nucleosomes loading at the HO-puro locus in unselected and reselected cells, and the role of Artemis. A) Graphical sequence alignment of representative clones along the T7ST-Puro1 sequence - obtained with "Align 2" of NCBI BLAST. B) Histone H3 occupancy in unselected and reselected cell by ChiP. Chromatin was formaldheide cross-linked from 10^6 cells and ChIP for H3 was carried out as described in [7]. Input dilutions are from DNA isolated from 5000 cells and 3-fold dilutions thereof. PIS is the CHIP protocol carried out with pre-immune serum. ¼ input DNA is product generated by cross-link reversal from ¼ the material used for ChIP. Amp/Puro is the band generated with primers Amp1 and Puro1. C) Western blot for Artemis was carried out from reselected cells, before and after 24h treatment with siRNA against Artemis (purchased from Dharmacon). The blot was sequentially probed for Hsp90 and ATM. All antisera were from Abcam. D) Kinetics of cleavage and repair (HO time course of infection) with reselected cells treated or not with Artemis siRNA.

clearly possible, in reality the organization of nucleosomes in the two cell populations may be different, and hence produce bands that are only similar in size. A last caveat that we must suggest is that the PCR products may be to some extent an artifact. While it seems clear that they do represent secondary cleavage events after the main HO cut, it is possible that the PCR conditions would favor the amplification of only a few products, and that in reality the nucleolytic processing in the population is more heterogeneous. Multiplex PCR is very efficient, and up to 5 products have been independently and reliably amplified in published research. However, only one primer pair was used here, and hence the possibility of preferential amplification remains.

The addition of WMN during the time course of infection did not alter much the kinetics of cutting and repair, but the smaller bands appeared more defined and somewhat less smeary. We discuss later how this may be the effect of a shift in the activity of Artemis toward greater endonucleolytic performance and less exonucleolytic.

Evidence for formation of repressive chromatin was obtained by showing that expression of the puro mRNA was reduced in unselected cells, and then restored to higher level after reselection. To seek direct evidence for a more condensed chromatin in the unselected cells compared to reselected, we determined by ChIP the histone H3 occupancy at the HO cassette. To obtain a larger snap-shot of the region and maximize the difference in nucleosomal load between unselected and reselected cells, primers Amp1 and Puro1 were used, which encompass a ~2 kb segment. The results of a representative experiment are shown in Figure 2B, which revealed a ~3-fold stronger ChIP signal from unselected vs. reselected cells. The data were compared to the signal obtained from the FABP amplicon, which resides in a gene that is not transcriptionally active in these cells. These data further support the idea that the region spanning the engineered HO-puro cassette had become heterochromatic and silenced, hence more stably loaded with nucleosomes, in the absence of selective pressure. Additional evidence for this could come from determination of repressive chromatin marks (e.g., histone H3K9me3 or DNA methylation) in future experiments.

The involvement of Artmeis in generating the repair ladder was hypothetical, somewhat corroborated by the enhancing effect of WMN in this experiment. Clearly, there was some downstream effect from inhibition of one or more PIKK, and Artemis is known to be regulated by both ATM and DNA-PK. It was also reported that *in vitro* the endonucleolytic and exonucleolytic activities of Artemis are oppositely regulated by DNA-PK [5]. We thus decided to test if Artemis, which is not an essential protein, was in fact involved in this process. Knockdown of Artemis was induced with siRNA, and 80% reduction was obtained with 100 nM of oligo (Figure 2C). The same blot was also probed for ATM to verify presence of this critical protein in the process, and Hsp90 as loading control. We then pre-treated the cells for one day with siRNA and repeated the infection with Adeno-HO with selected time points. Without the siRNA, the experiment gave essentially the same results as in Figure 1B, with again a prominent band of ≤500 bp. There was a hint of bands 1 and 3, more noticeable at the 3h PI early time point, but since they are less intense than those in Figure 1B, it suggests that ends-processing may be a stochastic process, possibly dictated by the predominant chromatin organization in the population at the time of the experiment. The two experiments were carried out within two weeks of each other - 4 passages. In cells knocked down for Artmeis, there was instead an indistinct smear of products between ~600 and 200 bp. Hence, while clearly there was nucleolytic activity near the DSB, there was no evidence for generation of a distinct laddering pattern. This experiment suggested that Artemis may be involved in a specific pattern of ends-processing at the DSB, consisting of internucleosomal, endonucleolytic release of fragments adjacent to the HO cut site before religation. This pattern was somewhat enhanced by inhibition PIKKs, which presumably alters the specificity of Artemis. In the absence of Artemis (knock-down experiment) the ends are apparently processed more heterogeneously, likely by a combination of other endo- and exo-nucleases. For instance, the Mre11 nuclease component of the DSB-binding complex MRN could provide such function [10]. While this paper was undergoing review, Helmink et al. reported that presence of γH2AX in chromatin flanking the DSBs generated by the RAG recombinase during V(D)J recombination affects ends resection and processing by Artemis and CtIP [11].

Conclusions

In this paper we report a few new findings using a system adapted from yeast to introduce a single genomic DSB in mammalian cells. First, as in yeast, the cleavage at the genomic site appears influenced by the organization of chromatin. It is more accessible when the site is euchromatic, and less accessible when the locus becomes more heterochromatic and transcriptionally silent. Second, in the reselected cells, we identified a novel pattern of processing and repair at the junctions, which generated a curious and prominent PCR ladder, which we interpret as removal of DNA segments bound to nucleosomes or other protein complexes. If we are right, first, the nucleosomes must be positioned in the majority of the cells for this to occur. Second, this is clearly different from the pattern of accurate repair originally seen when the first passages of MM3MG-HO cells were analyzed. In those cells, there was one prominent PCR product identical in size and sequence to that of uncut cells. It is tempting to speculate that in the original cells, the locus was even less packed with nucleosome, although histones were clearly present [7]. Alternatively, the nucleosomes were more readily evicted during DSB repair [7]. We hypothesize instead, that in the reselected cells, the repair now requires more extensive remodeling of the locus and the resection of adjacent nucleosomes, and that this happened after the establishment of repressive chromatin in the absence of selection. The involvement of Artemis in the internucleosomal endonucleolytic cleavage at either side of the DSB is also a novel issue, *albeit* incompletely resolved. Artemis was previously reported to be partly responsible for generating the apoptotic ladder [6], and in fact, cells lacking Artemis lack or show delayed kinetics of appearance of the typical banding induced by apoptosis [6]. We suggest that Artemis is responsible for producing the ladder we observe in our reselected cells, and that this activity might be important to create short segments of DNA ends that are better suitable for religation. The role of chromatin at DSBs is clearly a known example of how Artemis may be particularly involved in processing of perhaps only a fraction of IR-induced DSBs, which are however localized to heterochromatin [3,12]. We should point out, however, that Goodarzi et al. suggested that Artemis' action is particularly involved in HRR during the G2 phase of cell cycle [12]. In contrast, our work suggests that Artemis is clearly involved in NHEJ and in unsynchronized cells (hence largely in G1-S). It was possibly a lucky accident that the original clone of puromycin-selected cells with that specific HO integration site, which we ended up selecting for our studies, displayed plasticity in chromatin organization at the locus.

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Authors' contributions

PKM and ADB contributed Figure 1. SR and ADB contributed Figure 2. ADB wrote the paper with the help of PKM and SR. All authors have read and approved the manuscript.

Competing interests

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SHORT REPORT Open Access

Tousled kinase TLK1B mediates chromatin assembly in conjunction with Asf1 regardless of its kinase activity

Arrigo De Benedetti

Abstract

Background: The Tousled Like Kinases (TLKs) are involved in chromatin dynamics, including DNA replication and repair, transcription, and chromosome segregation. Indeed, the first two TLK1 substrates were identified as the histone H3 and Asf1 (a histone H3/H4 chaperone), which immediately suggested a function in chromatin remodeling. However, despite the straightforward assumption that TLK1 acts simply by phosphorylating its substrates and hence modifying their activity, TLK1 also acts as a chaperone. In fact, a kinase-dead (KD) mutant of TLK1B is functional in stimulating chromatin assembly in vitro. However, subtle effects of Asf1 phosphorylation are more difficult to probe in chromatin assembly assays. Not until very recently was the Asf1 site phosphorylated by TLK1 identified. This has allowed for probing directly the functionality of a site-directed mutant of Asf1 in chromatin assembly assays.

Findings: Addition of either wt or non-phosphorylatable mutant Asf1 to nuclear extract stimulates chromatin assembly on a plasmid. Similarly, TLK1B-KD stimulates chromatin assembly and it synergizes in reactions with supplemental Asf1 (wt or non-phosphorylatable mutant).

Conclusions: Although the actual function of TLKs as mediators of Asf1 activity cannot be easily studied in vivo, particularly since in mammalian cells there are two TLK genes and two Asf1 genes, we were able to study specifically the stimulation of chromatin assembly in vitro. In such assays, clearly the TLK1 kinase activity was not critical, as neither a non-phosphorylatable Asf1 nor use of the TLK1B-KD impaired the stimulation of nucleosome formation.

Background

The anti-silencing factor Asf1 was originally identified as a protein that when overexpressed derepressed the silent mating loci on chromosome III of *S. cerevisiae* [1]. These are well-characterized heterochromatic regions that, like those of telomeres and rRNA gene clusters, are transcriptionally repressed. It was later found that Asf1 is a histone H3/H4 chaperone [2] that, in conjunction with other factors like CAF1 and HIRA, can mediate both chromatin assembly and disassembly [3] during replication [4-6], transcription [7-9], and DNA repair [10-14]. It is likely that Asf1 mediates its effects by inducing localized or global chromatin remodeling, depending on the situation. Given all of these functions, it is not surprising that Asf1 is essential in mammalian cells [15] and other organisms [16], including *S. pombe* [17], but actually not in *S. cerevisiae*, although budding yeast deleted for Asf1 are sensitive to genotoxins and display elevated chromosomal instability [18]. Very recently it was found that Asf1, in conjunction with Rtt109, plays an important role in preventing replication errors at repetitive sequences in budding yeast [19]. Asf1 can be found in association with many other proteins, frequently in organism/tissue-specific fashion [20], consistent with its many molecular and developmental functions [16]. In addition to its function as a direct histone H3/H4 chaperone, Asf1 also mediates modifications of histone marks [20,21,13].

The gene *Tousled* of *Arabidopsis thaliana* encodes a protein kinase which, when mutated, results in

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abnormal flower development [22]. This was proposed to be linked to a replicative defect during organogenesis [23], but which may also result from failure to protect the genome from UV damage [24,12], resulting in mitotic aberrations [25-27]. Two *Tousled* genes (TLK1 and TLK2) with several splice variants were identified in mammals [28,29], and were confirmed as encoding kinases. Few physiologic substrates of *Tousled like kinases* (TLKs) have been identified, namely Asf1 [30], histone H3 [31], Aurora B [26], and more recently Rad9 in mammalian cells [32] and two mitotic kinesins in Trypanosomes [33]. This immediately suggested a function in chromatin assembly [34] during transcription [35,24], DNA repair [12,36], and condensation and segregation of chromosomes at mitosis [25]. Interestingly, all of these substrates were identified via their tight interaction with TLK, and not by classic kinase assays that usually reveal transient kinase-substrate interactions. In fact, where investigated, the association of TLK with its substrates is not ablated when a kinase-dead (KD) mutant of the protein is used [30,37,26], and in many cases it can promote functional effects in defined assays, and whether ATP is present or not [26,38,37]. This is certainly true for the binding of TLK1 with Asf1 [30,38], and in fact, the identification of hAsf1 as a substrate of TLK1 came about from a two-hybrid screen and was then confirmed with in vitro pull-downs with wt or TLK1-KD. The actual phosphorylation site was unknown at the time [30] and not identified until very recently [39]. Moreover, TLK1 can promote repair of DSBs generated with radiation [31,36] despite the fact that it is known that the actual kinase activity is inhibited due to genotoxic stress [40,41] via a DNA damage checkpoint relay [40]. This phenomenon may implicate aspects of chromatin remodeling that depend on TLK1 and Asf1 after radiation [34]. Hence, it was not very clear what could be the role of Asf1 phosphorylation by TLK, if any. Since the recent identification of the Asf1 phosphorylation site(s), in human and Drosophila, it has become possible to ascertain the potential significance of this phosphorylation. Perhaps disappointingly but maybe not surprisingly, the only effect that was reported from site-directed abrogation of the phosphorylation site in hAsf1a (and dAsf1) was an increased turnover of the protein, while hAsf1b stability was not dependent at all on phosphorylation [39]. No phenotypic effects were reported by the authors for the site-directed Asf1 mutants expressed in cell lines, and most likely there weren't any. First, Asf1 proteins are generally abundant, and even the modest (~50%) reduction reported is unlikely to result in significant effects in general aspects of chromatin dynamics. Second, at least in the case of man, there are two redundant Asf1 proteins (Asf1 and Asf1b), and only the stability of Asf1a was affected. Nonetheless, without the actual replacement of the two hAsf1 genes by site-directed mutants, it is not possible to establish the role of TLK-mediated phosphorylation in vivo. Asf1 is phosphorylated in *S. cerevisiae* (Jessica Tyler, personal communication), and of course, Asf1 replacements are easy to make in yeast, but this organism lacks TLKs. Thus, we are left with few options for establishing the role of such phosphorylation in higher organisms, in cell lines and even more importantly in transgenic animals. However, we have developed in vitro assays of chromatin assembly in mammalian extracts [12,36,14], and these assays revealed a dependence on TLK1 and Asf1a. In these assays, chromatin assembly was stimulated by the addition of wt or TLK1B-KD [14,37], but whether this effect required the presence of Asf1 or more importantly its phosphorylation was unclear. As the extract-mediated chromatin assembly in vitro requires ATP, it was not possible to dissect the specific role of Asf1 phosphorylation by TLK1. With the new information on the Asf1 phosphorylation site by TLK1 [39], it has now become possible to address the potential role of this phosphorylation at least for chromatin assembly in vitro. We found that phosphorylation of hAsf1a at S192 is not essential for nucleosome assembly.

Findings and Discussion

In vitro phosphorylation of Asf1 wt and mutant

We have expressed wt hAsf1a and the mutant, Asf1 (S192Y), and carried out an in vitro phosphorylation with hTLK1B and [γ32P]ATP. We confirmed that S192 is the major phosphorylation site (Figure 1A). With this mutated Asf1, it became possible to study whether TLK1B acts as a kinase and/or as a chaperone for Asf1 in chromatin assembly.

TLK1B stimulates chromatin assembly in vitro regardless of kinase activity and Asf1 phosphorylation

We have previously described a cell-free system in which the addition of TLK1B enhances the assembly of chromatin by an in vitro plasmid supercoiling assay, and that this depends on presence of Asf1 [12]. In this assay, Bluescript plasmid isolated from bacteria was first relaxed with wheat-germ Topoisomerase 1 (Figure 1 panel B, lane 5 and panel C, lane 7). After repurification with Geneclean, this was used as a template for the deposition of core histones in the presence of MM3MG nuclear extract and an energy mix. The extract causes the relaxed form to convert to supercoiled topoisomers of faster mobility, since some plasmid becomes bound in nucleosomes and migrates as a series of discrete forms due to a decrease in the linking number (i.e., one negative supercoil per nucleosome). The extract contains all the factors needed for chromatin assembly,

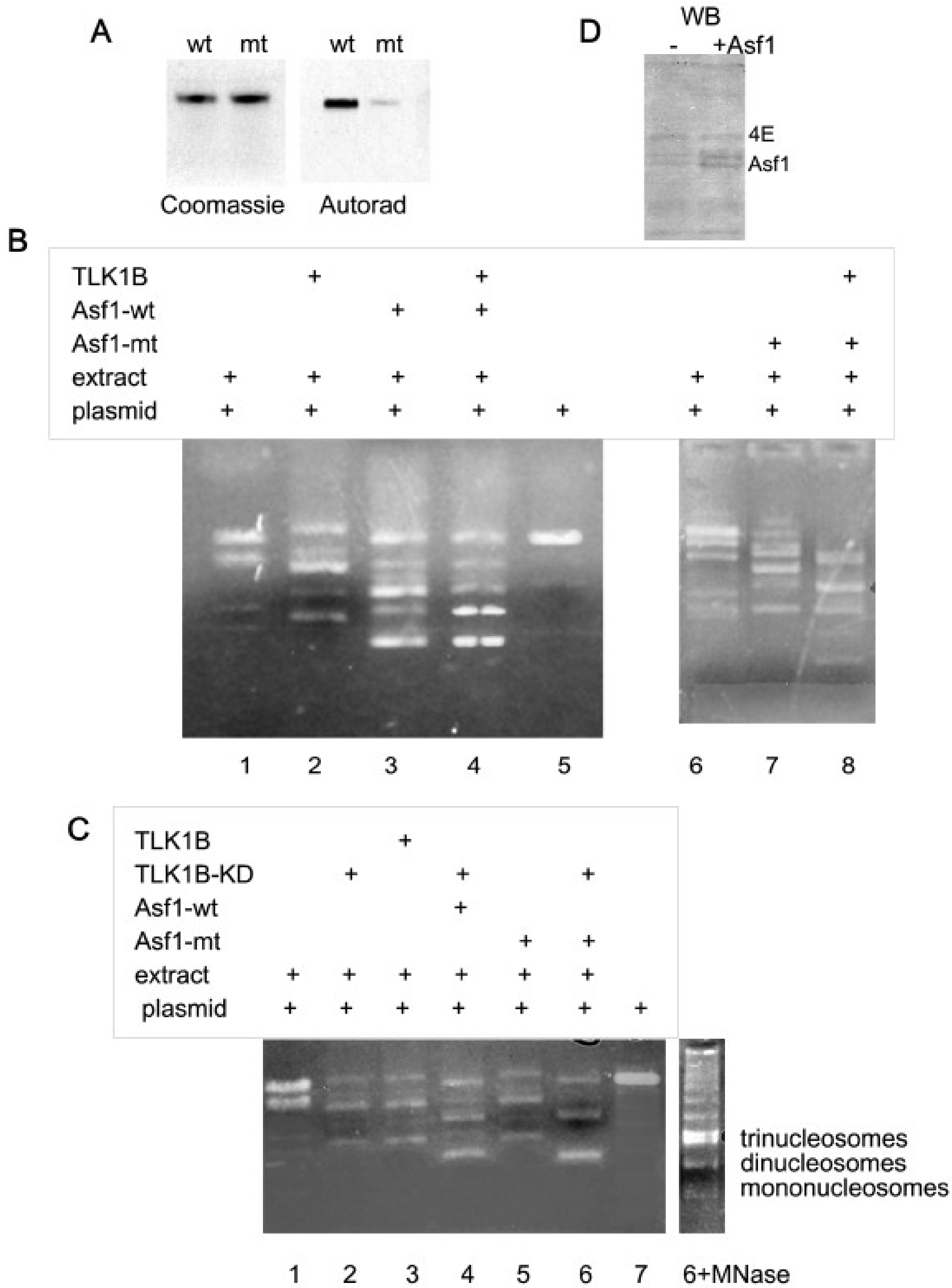

Figure 1 TLK1B mediates chromatin assembly with Asf1 regardless of Asf1 phosphorylation. **A**. In vitro phosphorylation of Asf1 wt and S192Y mutant by TLK1B. **B**. Chromatin assembly on a plasmid is stimulated equally well by Asf1 wt and mutant, and the addition of wt TLK1B further stimulates plasmid supercoiling. **C**. Chromatin assembly is stimulated by TLK1B, wt or KD, and the addition of wt or mutant Asf1 synergizes in plasmid supercoiling. **D**. Western blot for Asf1a in nuclear extract supplemented or not with recombinant Asf1a. The blot was also probed for eIF4E (4E) as a loading control.

including topoisomerases and core histones. The addition of Asf1, wt or mutant, resulted in stimulation of supercoiling to the same degree (Figure 1B, lanes 3 and 7). The addition of recombinant TLK1B stimulated the formation of the more highly supercoiled forms, and a similar effect was obtained for the mutant Asf1, clearly indicating that the mutant Asf1 is proficient in chromatin assembly and that TLK1B can stimulate this process in the absence of kinase activity.

To further test the idea that it is the chaperone activity of TLK1B, and not its kinase function that promotes stimulation of chromatin assembly via stimulation of Asf1, the experiment was repeated using the TLK1B-KD. Interestingly, the same activity was seen with addition of the wt or KD protein, indicating that stimulation of chromatin assembly does not depend on the kinase activity (Figure 1C). The simultaneous addition of wt or TLK1B-KD and Asf1 (wt or non-phosphorylatable mutant) synergized in the formation of more highly supercoiled plasmid forms. To confirm that the supercoiled, high-mobility forms are indeed due to formation of nucleosomes, the end-products of the reaction were subjected to MNase treatment, which resulted in the typical nucleosomal ladder (panel C, lane 6+MNase). We have previously shown that depletion of Asf1 from the nuclear extract does not preclude formation of chromatin, but that this occurs more slowly during a time course [12,14]. Hence, the addition of TLK1B and Asf1 to the extract clearly stimulates the assembly of nucleosomes, but this does not depend on TLK1B kinase activity. Rather it depends on its chaperone function. Although we should caution that endogenous Asf1a and Asf1b are obviously present in the nuclear extract, somewhat complicating the interpretation of the results, the mutant hAsf1a was added to a level ~3-fold greater than the endogenous level (western blot, panel D), and hence, should be in the optimal range as a competitor. Nonetheless, clearly these studies rely on ectopically added proteins above the endogenous level, and hence have limitations.

Conclusions

Mutants of *Tousled* were originally identified in *A. Thaliana* based on a phenotype manifested as stunted organogenesis [22,23]. While it was assumed that the phenotype was caused by loss of the kinase activity, this was not directly determined for the specific mutants identified, and it is entirely possible that it is instead the chaperone activity of *Tousled* that is affected. In fact, it was immediately recognized that hTLK1-KD was able to bind well to hAsf1 [30]. Moreover, expression of TLK1B-KD in cells, or addition of excess mutant protein in chromatin assembly in vitro, resulted in stimulation of nucleosome formation [37,14]. Although the actual function(s) of TLKs as mediators of Asf1 activity cannot be easily studied in vivo, the recent identification of the hAsf1a phosphorylation site [39] has allowed for direct analysis of the significance of this phosphorylation in chromatin assembly in vitro. The experiments reported here suggest that the phosphorylation of Asf1 is not very significant for explaining the stimulation of TLK1B on chromatin assembly in vitro. And although there could be other activities of TLK1B (wt or KD) that may have been overlooked, such as a potential effect on histone H3 [31] or the potential formation of kinase-active dimers between wt and KD protein, the ability to detect stimulation of nucleosome formation by excess non-phosphorylatable Asf1 indicates that the role of TLK1 as a kinase for Asf1 is not very critical in these reactions. Another possible caveat is that TLK kinase activity and the phosphorylation of at least a small amount of Asf1 may still be important to initiate the process of chromatin assembly, but once started, the function of TLK1 could be less catalytic and more like a chaperone. It would be very difficult to address this caveat without the complete depletion of Asf1 and all the isoforms of TLK1 and TLK2.

We conclude that the kinase activity of TLK1B for Asf1 is not a key determinant as a modulator of Asf1 activity. Further support to the idea that TLK1 may act as a molecular chaperone for its substrates comes from its reported interaction with Aurora B in *C. elegans* [26]. TLK1 increased the activity of Aurora B in vitro in a manner that was independent of its kinase function, again suggesting that the kinase activity of TLK1, although certainly important, is insufficient to explain all its functions. Further, TLK1B-KD was proficient as the wt protein in recruiting Rad9 to a DSB in vivo, and TLK1 was found tightly associated with the chromosomal passenger complex and with two mitotic kinesins in Trypanosomes [33]. Whereas typically, most protein kinases do not bind their substrates very tightly and have only a transient interaction, TLKs bind their substrates very tightly and copurify in large complexes [41], which suggests additional activities. Also, mammalian TLK1 is a rather abundant protein, and since protein kinases have usually high turnover rates of their substrates, it seems puzzling that such a high expression would be needed based simply on a kinase role, while a function as a chaperone could explain this (recall that histone H3 and Asf1 are abundant). All of these observations suggest that TLKs are rather chaperones [14].

Methods

Preparation of nuclear extract and chromatin assembly in vitro were as described in [12,14]. The reactions were incubated for 30 min, which is sufficient to reach equilibrated deposition of histones on the plasmid template.

Purified GST-TLK1B (wt and KD) were prepared as described in [37], and hAsf1a (CIA) was purified as described in [12]. The Asf1 unphosphorylatable mutant was generated with the oligo: 5'-gaaaact***acg***taaatgtcatgt-tagaatccc, changing S^{192} and L^{193} to Y, V, which also introduced a diagnostic SnaBI site. The mutation was confirmed by sequencing. The recombinant proteins were added at 50 nM final concentration.

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Authors' contributions
ADB is solely responsible for this work.

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